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Human Body Fluid Ions In colchicine complexes ESI MS, MADLI MS, Spectroscopic, DFT Studies and Fungicidal Activity of colchicine complexes With Sodium, Potassium, Magnesium and calcium carbonates and Sulphates

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Abstract: Colchicine is an alkaloid characterised by good water solubility. After administration of colchicine as a medicine for example for the treatment of gout, colchicine probably forms some more or less stable structures with cations and/or anions present in human body fluid. The colchicine complexes with Na^+, K^+ Mg^{2+} and Ca^{2+} cations of sulphates and carbonates have been synthesized and studied by ESI MS, MALDI MS, ^1H and ^{13}C NMR, FT IR DFT calculations and also have been tested against fungicidal activity. Salts of good solubility in water have been chosen, like Na_2SO_4, K_2SO_4, Na_2CO_3, K_2CO_3, $MgSO_4$ and $CaSO_4$. It has been shown that colchicine forms stable complexes of 1:1 stoichiometry with monovalent and divalent metal cations. For K^+ and Na^+ cations also formation of 2:1 stoichiometry complexes has been detected. Colchicine with sodium sulphate forms much more complicated structures of 1:2:1 and 2:2:1 stoichiometry in which sulphate anion is involved. Colchicine complexes have fungicidal activity.

Keywords *- complexes of colchicine with monovalent and divalent metal cations, DFT, fungicidal activity of colchicine complexes, spectroscopic studies (ESI MS, MALDI MS, NMR, FT IR)*

I. INTRODUCTION

Colchicine **1** (Fig.1.) is a tropolone alkaloid of *Colchicum autumnale.* It shows antimitotic, antifibriotic, anti-inflammatory activity [1] and can efficiently exacerbate the symptoms during an attack of gout when applied in the early phase. More recently it has been introduced in the treatment of familiar Mediterranean fever [2]. Moreover, colchicine **1** is a potent anti-mitotic agent and shows carcinogenic activity [4-6]. Similarly to other alkaloids, colchicine **1** can act through blocking or activating of specific receptors or ion channels in living organisms. Its activity depends on the ability of formation of noncovalent complexes with macromolecules such as tubulin in microtubules.

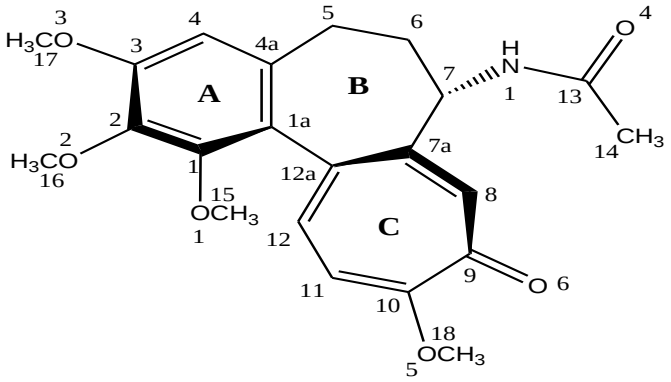


Fig. 1. Colchicine molecule, carbon and oxygen atoms numbering

In human body fluid (extracellular fluid, transcellular fluid, blood, cytosol and saliva) some electrolytes are present, in particular sodium, potassium, magnesium and calcium cations. Electrolytes like Na^+, Ca^{2+}, Mg^{2+}

and anions Cl^-, HCO_3^{2-}, SO_4^{2-}, $H_2PO_4^-$, HPO_4^{2-}, play a vital role in intravascular osmotic effect that keeps electrolytes in balanced form and protects the body from infection and other blood disorders. [7-13] In order to maintain osmotic balance, the compartments of a mammal's body must be able to excrete and absorb water to and from the environment. Exchange of respective ions cause the osmotic system in the body. The ions cooperate in keeping constant pH of the environment because some of them are components of buffers. Inorganic ions must also be exchanged between extracellular fluid and the external environment to maintain homeostasis. It has been established that the rate of binding of colchicine to tubulin is enhanced by certain anions. Among the inorganic anions tested, only sulphate was effective. [14] In contrast to extracellular fluid, cytosol has a high concentration of potassium ions and a low concentration of sodium ions.[11] This difference in ion concentrations is critical for osmoregulation, since if the ion levels were the same inside and outside of the cell, water would enter constantly by osmosis - since the concentrations of macromolecules inside the cells are higher than their levels outside. The loss of sodium and chloride ions compensates for the osmotic effect of the higher concentration of organic molecules inside the cell. [8-14]

Not much research has been made on the formation of complexes between colchicines and cations. Only Mackay et al. in 1998 obtained a derivative of colchicine -hydrated crystals of copper(II) colchiceine (10-hydroxycolchicine) that belongs to the tetragonal space group [15].

In the previous work we reported results of a study on coordination process of colchicine **1** with iodides and perchlorates of monovalent metal ions (lithium, sodium and potassium salts) [16]. Some theoretical study has been made on coordination process of colchicine - Na^+ cation.[17] Other biologically active compounds like: lasalocid polyoxalkyl esters [18, 19], derivatives of gossypol [20] or oligomycin A [21, 22] also were investigated as ligands in complexation process with monovalent metal ions Li^+, Na^+ and K^+, Cs^+ or Rb^+.

To the best of our knowledge, the process of complexation of colchicine **1** with monovalent and divalent metal ions present in human body fluid such as: Mg^{2+}, Ca^{2+}, Na^+ and K^+ with respective anions: sulphate and carbonate has not been studied yet. This fact has prompted us to obtain and examine the structure of colchicine complexes **2-7** with water soluble sulphates and carbonates with monovalent cations: Na^+ and K^+ and divalent cations: Mg^{2+} and Ca^{2+}.

The aim of this study was the synthesis of colchicine coordinative compound with inorganic salts: carbonates and sulphates (sodium, potassium, magnesium and calcium) which show good water solubility. This study is a continuation of research work on the coordination process of colchicine and monovalent metal cations that could also play a biologically important role. New derivatives **2-7** were characterized by MS (ESI and MALDI), FT IR, ^{1}H NMR and ^{13}C NMR and subjected to theoretical studies.

Moreover, to the best of our knowledge, these compounds are for the first time in our study tested towards the fungistatic activity of the microfungi species (*Aspergillus niger* van Tiegen, *A. versicolor*, *Paecilomyces variotii*, *Penicillium funiculosum*) using the method of bioautography on thin-layer plates.[23] Some complexes were selected from among the new derivatives and tested against brown-rot fungi *Coniophora puteana* and *Poria placenta* and also against white-rot fungus *Coriolus versicolor* using agar and broth dilution method to determine the minimal inhibitory concentration (MIC). Colchicine itself has been tested against *A. niger* previously and it did not show fungistatic properties against this fungus.

The structures of colchicine complexes **2-7** with salts of divalent (Mg^{2+}, Ca^{2+}) and monovalent (Na^+ and K^+) metal cations are discussed. The above-mentioned ions as well as sulphate and carbonate anions are present in the human body fluids. On the basis of experiments performed and the analysis of FT IR, ^{1}H NMR, ^{13}C NMR, ESI MS and MALDI MS spectra as well as the elementary analysis and theoretical studies of these compounds, colchicine was demonstrated to have complexing capacity of the ions mentioned.

II. MATERIAL AND METHODS

2.1. MaterialsColchicine **1** is commercially available on ApplyChem. For complexation reaction a natural isomer (-)-(a*R*, 7*S*) of colchicine was used. Salts Na_2CO_3, Na_2SO_4, K_2SO_4, K_2CO_3, $MgSO_4$ and $CaSO_4$ from Sigma-Aldrich were obtained commercially and used without any purification. Solvents used for the synthesis were purified by standard methods.

2.2. Measurements

The NMR spectra of colchicine and its 1:1 complexes (0.07 mol L^{-1}) with monovalent and divalent metal cations salts were recorded in CD_3CN solutions using a Varian Gemini 300 MHz spectrometer. All spectra were locked to deuterium resonance of CD_3CN. The ^{1}H NMR measurements in CD_3CN were carried out at the operating frequency 300.075 MHz; flip angle, pw = 45^0; spectral width, sw = 4500 Hz; acquisition time, at = 2.0 s; relaxation delay, d_1=1.0 s; T = 293.0 K and using TMS as the internal standard. No window function or zero filling was used. Digital resolution was 0.2 Hz per point. The error of chemical shift value was 0.01 ppm. ^{13}C NMR spectra were recorded at the operating frequency 75.454 MHz; pw = 60^0; sw = 19000 Hz; at = 1.8 s; d_1=1.0 s; T = 293.0 K and TMS as the internal standard. Line broadening parameters were 0.5 or 1 Hz. The error

of chemical shift value was 0.01 ppm. The ^{1}H and ^{13}C NMR signals were assigned for each species using one or two-dimensional spectra. The FT IR spectra of colchicine and its 1:1 complex (0.07 mol dm^{-3}) were recorded in the mid infrared region in KBr pellets using a Bruker IFS 113v spectrometer. A cell with Si windows and wedge-shaped layers was used to avoid interferences (mean layer thickness 170 μm). The spectra were taken with an IFS 113v FT-IR spectrophotometer (Bruker, Karlsruhe) equipped with a DTGS detector; resolution 2 cm^{-1}, NSS = 125. The Happ-Genzel apodization function was used. All manipulations with the substances were performed in a carefully dried and CO_2-free glove box. The UV-Vis spectra were recorded in methanol by means of JASCO V-550 spectrophotometer at 200-600 nm of measurement range. The ESI (Electrospray Ionisation) mass spectra were recorded on a Waters/Micromass (Manchester, UK) ZQ mass spectrometer equipped with a Harvard Apparatus syringe pump. All samples were prepared in CD_3CN. The measurements were performed for the samples being the solutions of colchicine complexes: colchicine concentration ($5x10^{-5}$mol dm^{-3}) with: each of the cations Na^+, K^+, Ca^{2+} and Mg^{2+} ($2.5x10^{-4}$ mol dm^{-3}) were recorded separately. The samples were infused into the ESI source using a Harvard pump at a flow rate of 20μl min^{-1}. The ESI source potentials were: capillary 3kV, lens 0.5kV, extractor 4V. The standard ESI mass spectra were recorded at 30V. The source temperature was 120°C and the desolvation temperature was 300°C. Nitrogen was used as the nebulizing and desolvation gas at flow-rates of 100 and 300dm3 h-1, respectively. Mass spectra were acquired in the positive ion detection mode with unit mass resolution at a step of 1 *m/z* unit. The mass range for ESI experiments was from *m/z* = 100 to *m/z* = 1400. The elementary analysis of colchicine complexes **2-7** were carried out on Vario ELIII (Elementar, Germany).

The matrix-assisted laser desorption/ionization measurements, including MS/MS experiments, were accomplished on Waters Q-TOF Premier instrument, equipped with nitrogen laser MALDI source and MassLynx™ software. MALDI measurements were performed in the positive ion mode. In order to prepare the target spots, the methanolic solution containing matrix (1 μl, concentration 0.5 mol/dm^3) was deposited on the spot and allowed to dry at room temperature. After a few minutes 1 μl of solution containing respective colchicine complexes (the concentration about 1 mmol/dm^3) was placed as a next layer over the dried matrix and left to cocrystallize. The MS/MS experiment with dithranol as a matrix and argon as colliding neutral gas (at flow rate of 0.5 mL/min) was performed and the collision-induced fragmentation of protonated molecules $[M+H]^+$ was analyzed, depending on collision energy CE (Table 2). The product ion MS/MS spectra were collected at five collision energy values, i. e. 20, 30, 40, 50 and 60 eV. It should be noticed the nanospray MS of colchicine and MS^2 of the m/z 400±0.5 ion $[M+H]^+$ have already been reported and representative fragment ions, which originate from m/z 400, are given, i.e. m/z 382, 368, 358, 341, 326, 310, 298 and 282. The same m/z values appear in collisionally induced dissociation as well as in previously published papers.[24-26]

2.3. Preparation of complexes

Complexes of colchicine **2-7** were obtained by dissolving of the respective salts and colchicine in the 1:1 ratio in methanol. Complexes were prepared from colchicine (96 mg, 0,25 mM) and sodium carbonate (27 mg, 0,25 mM), potassium carbonate (35 mg, 0,25 mM), sodium sulfate (36 mg, 0,25 mM), potassium sulfate (44 mg, 0,25 mM), magnesium sulfate (30 mg, 0,25 mM), calcium sulfate (34 mg, 0,25 mM), respectively. The solution was evaporated until the product started to precipitate. The resulting precipitate was filtered off and recrystallized from ethanol. The purity of all the complexes was checked by TLC. Carbon atom numbering of colchicine **1** is shown in Fig. 1.

For complex **[**$Na_2(C_{22}H_{25}NO_6)CO_3$**]** **2**: Yield 93%, 117 mg; M. p. = 105-107°C, *Anal.* Calc. 49.32 C; 5.54 H; 2.50 N Found 48.84 C; 6.04 H; 2.53 N $C_{23}H_{25}NO_9Na_2 \cdot 3H_2O$. UV vis ($CH_3OH$) λ_{max} [nm] 351, 244, ^{1}H NMR (300 MHz, CD_3CN, TMS, ppm, 25°C): 6.69 (HC-4, s), 2.33, 2.55 (H_2C-5), 1.83, 2.11 (H_2C-6), 4.37 (HC-7), 7.24 (HC-8, s), 6.94 (HC-11, d, *J* = 10.7 Hz), 7.17 (HC-12, d, *J* = 10.7 Hz), 3.61 (H_3C-15, s), 3.86 (H_3C-16, s), 3.83 (H_3C-17, s), 3.91 (H_3C-18, s), 1.88 (H_3C-14, s), 7.36 (NH, d, *J* = 7.00 Hz); ^{13}C NMR (75 MHz, CD_3CN, TMS, ppm): 151.72 (C-1), 126.57 (C-1a), 142.14 (C-2), 154.44 (C-3), 108.57 (C-4), 136.74 (C-4a), 30.27 (C-5), 36.86 (C-6), 52.99 (C-7), 152.05 (C-7a), 131.37 (C-8), 179.63 (C-9), 164.89 (C-10), 113.09 (C-11), 136.74 (C-12), 135.59 (C-12a), 61.65 (C-15), 61.42 (C-16), 56.62 (C-17), 56.80 (C-18), 170.26 (C-13), 22.72 (C-14). IR (KBr): 3403, 3273, 2936, 2838, 1662 (υC=O), 1615 (υC=O), 1589, 1558, 1487, 1431, 1284, 1253, 1138, 1093, 841 (CO_3^{2-}).

For complex **[**K_2**(**$C_{22}H_{25}NO_6)CO_3$] **3**: Yield 96%, 129 mg; M. p. = 182-184°C, *Anal.* Calc. for $C_{22}H_{25}NO_6 \cdot K_2CO_3 \cdot 0.5H_2O$ 50.55 C; 4.56 H; 2.56 N %, Found 49.68 C; 4.16 H; 2.41 N. UV vis (CH_3OH) λ_{max} [nm] 352, 244; ^{1}H NMR (300 MHz, CD_3CN, TMS, ppm, 25°C): 6.72 (HC-4, s), 2.26, 2.55 (H_2C-5), 1.90, 2.26 (H_2C-6), 4.37 (HC-7), 7.18 (HC-8, s), 6.93 (HC-11, d, *J* = 10.7 Hz), 7.18 (HC-12, d, *J* = 10.6 Hz), 3.61 (H_3C-15, s), 3.86 (H_3C-16, s), 3.86 (H_3C-17, s), 3.95 (H_3C-18, s), 1.90 (H_3C-14, s), 7.36 (NH, d, *J* = 7.00 Hz); ^{13}C NMR (75 MHz, CD_3CN, TMS, ppm): 151.73 (C-1), 126.59 (C-1a), 142.15 (C-2), 154.43 (C-3), 108.56 (C-4), 136.68 (C-4a), 30.29 (C-5), 36.86 (C-6), 52.97 (C-7), 151.99 (C-7a), 131.36 (C-8), 179.66 (C-9), 164.88 (C-10), 112.99 (C-11), 136.68 (C-12), 135.59 (C-12a), 61.63 (C-15), 61.41 (C-16), 56.61 (C-17), 56.79 (C-18), 170.12 (C-13),

22.76 (C-14). IR (KBr): 3421, 3272, 2936, 1658 (υC=O), 1616 (υC=O), 1589, 1559, 1487, 1285, 1253, 1139, 1093, 832 (CO_3^{2-}).

For complex $[Na_2(C_{22}H_{25}NO_6)SO_4]$ **4**: Yield 94%, 127 mg; M. p. = 150-153°C, *Anal.* Calc. for $C_{22}H_{25}NO_6 \cdot Na_2SO_4 \cdot 0.5H_2O$ 48.00 C; 4.72 H; 2.56 N, Found 48.16 C; 5.16 H; 2.44 N%. UV vis (CH_3OH) λ_{max} [nm] 351, 242; ^{1}H NMR (300 MHz, CD_3CN, TMS, ppm, 25°C): 6.69 (HC-4, s), 2.34, 2.55 (H_2C-5), 1.83, 2.12 (H_2C-6), 4.37 (HC-7), 7.23 (HC-8, s), 6.94 (HC-11, d, *J* = 11.2 Hz), 7.16 (HC-12, d, *J* = 10.7 Hz), 3.61 (H_3C-15, s), 3.86 (H_3C-16, s), 3.83 (H_3C-17, s), 3.91 (H_3C-18, s), 1.88 (H_3C-14, s), 7.35 (NH, d, *J* = 7.00 Hz); ^{13}C NMR (75 MHz, CD_3CN, TMS, ppm): 151.72 (C-1), 126.57 (C-1a), 142.13 (C-2), 154.43 (C-3), 108.57 (C-4), 136.74 (C-4a), 30.27 (C-5), 36.85 (C-6), 52.99 (C-7), 152.05 (C-7a), 131.34 (C-8), 179.70 (C-9), 164.87 (C-10), 113.08 (C-11), 136.74 (C-12), 135.62 (C-12a), 61.65 (C-15), 61.42 (C-16), 56.61 (C-17), 56.81 (C-18), 170.26 (C-13), 22.76 (C-14). IR (KBr): 3410, 3271, 2964, 2936, 1661 (υC=O), 1614 (υC=O), 1589, 1556, 1487, 1284, 1253, 1137, 1094.

For complex $[K_2(C_{22}H_{25}NO_6)SO_4]$ **5**: Yield 95%, 136 mg; M. p. = 153-155°C, *Anal.* Calc. for $C_{22}H_{25}NO_6 \cdot K_2SO_4 \cdot 0.5H_2O$ 48.53 C; 4.78 H; 2.57 N, Found 48.51 C; 4.00 H; 2.44 N%. UV vis (CH_3OH) λ_{max} [nm] 351, 243; ^{1}H NMR (300 MHz, CD_3CN, TMS, ppm, 25°C): 6.96 (HC-4, s), 2.24, 2.56 (H_2C-5), 1.81, 2.12 (H_2C-6), 4.36 (HC-7), 7.18 (HC-8, s), 6.92 (HC-11, d, *J* = 11.2 Hz), 7.16 (HC-12, d, *J* = 10.7 Hz), 3.61 (H_3C-15, s), 3.86 (H_3C-16, s), 3.83 (H_3C-17, s), 3.90 (H_3C-18, s), 1.88 (H_3C-14, s), 7.21 (NH, d, *J* = 7.00 Hz); ^{13}C NMR (75 MHz, CD_3CN, TMS, ppm): 151.74 (C-1), 126.60 (C-1a), 142.14 (C-2), 154.42 (C-3), 108.56 (C-4), 136.63 (C-4a), 30.28 (C-5), 36.85 (C-6), 52.95 (C-7), 151.88 (C-7a), 131.35 (C-8), 179.64 (C-9), 164.87 (C-10), 113.93 (C-11), 136.63 (C-12), 135.59 (C-12a), 61.63 (C-15), 61.41 (C-16), 56.60 (C-17), 56.78 (C-18), 170.12 (C-13), 22.74 (C-14), IR (KBr): 3433, 3275, 2936, 1663 (υC=O), 1615 (υC=O), 1589, 1558, 1487, 1284, 1252, 1138, 1093.

For complex $[Mg(C_{22}H_{25}NO_6)SO_4]$ **6**: Yield 93%, 121 mg; M. p. = 110-112°C, *Anal.* Calc. for $C_{22}H_{25}NO_6 \cdot MgSO_4 \cdot H_2O$ 49.16 C; 5.03 H; 2.61 N, Found 56.61 C; 6.64 H; 2.96 N%]. UV vis (CH_3OH) λ_{max} [nm] 351, 244; ^{1}H NMR (300 MHz, CD_3CN, TMS, ppm, 25°C): 6.96 (HC-4, s), 2.19, 2.56 (H_2C-5), 1.80, 2.10 (H_2C-6), 4.36 (HC-7), 7.16 (HC-8, s), 6.91 (HC-11, d, *J* = 10.6 Hz), 7.11 (HC-12, d, *J* = 10.7 Hz), 3.60 (H_3C-15, s), 3.86 (H_3C-16, s), 3.83 (H_3C-17, s), 3.90 (H_3C-18, s), 1.88 (H_3C-14, s), 7.19 (NH, d, *J* = 7.00 Hz); ^{13}C NMR (75 MHz, CD_3CN, TMS, ppm): 151.77 (C-1), 126.64 (C-1a), 142.19 (C-2), 154.43 (C-3), 108.58 (C-4), 136.58 (C-4a), 30.30 (C-5), 36.89 (C-6), 52.94 (C-7), 151.77 (C-7a), 131.37 (C-8), 179.61 (C-9), 164.88 (C-10), 112.85 (C-11), 136.58 (C-12), 135.59 (C-12a), 61.62 (C-15), 61.41 (C-16), 56.62 (C-17), 56.77 (C-18), 170.03 (C-13), 22.76 (C-14), IR (KBr): 3403, 3269, 2935, 1662 (υC=O), 1615 (υC=O), 1589, 1557, 1487, 1287, 1252, 1137, 1093.

For complex $[Ca(C_{22}H_{25}NO_6)SO_4]$ **7**: Yield 94%, 126 mg; M. p. = 157-159°C, *Anal.* Calc. for $C_{22}H_{25}NO_6 \cdot CaSO_4 \cdot H_2O$ 45.36 C; 4.46 H; 2.40 N Found 56.44 C; 6.28 H; 2.90 N %. UV vis (CH_3OH) λ_{max} [nm] 351, 244; ^{1}H NMR (300 MHz, CD_3CN, TMS, ppm, 25°C): 6.69 (HC-4, s), 2.33, 2.56 (H_2C-5), 1.81, 2.11 (H_2C-6), 4.36 (HC-7), 7.15 (HC-8, s), 6.91 (HC-11, d, *J* = 10.7 Hz), 7.15 (HC-12, d, *J* = 10.7 Hz), 3.61 (H_3C-15, s), 3.86 (H_3C-16, s), 3.83 (H_3C-17, s), 3.90 (H_3C-18, s), 1.88 (H_3C-14, s), 7.29 (NH, d, *J* = 7.00 Hz); ^{13}C NMR (75 MHz, CD_3CN, TMS, ppm): 151.81 (C-1), 126.64 (C-1a), 142.18 (C-2), 154.43 (C-3), 108.59 (C-4), 136.60 (C-4a), 30.30 (C-5), 36.89 (C-6), 52.95 (C-7), 151.81 (C-7a), 131.37 (C-8), 179.63 (C-9), 164.89 (C-10), 112.88 (C-11), 135.60 (C-12), 135.47 (C-12a), 61.62 (C-15), 61.42 (C-16), 56.62 (C-17), 56.78 (C-18), 170.05 (C-13), 22.76 (C-14), IR (KBr): 3433, 3274, 2934, 2838, 1663 (υC=O), 1615 (υC=O), 1589, 1558, 1487, 1284, 1253, 1139, 1093.

2.4. Quantum-mechanical calculations

All calculations were performed, within DFT framework at M06/SDD level of theory [27-28], selected on the basis of the results from extensive comparative studies of Zhao and Truhlar [27], recommended for calculations for compounds containing metal atoms [28-30]. Partial atomic charges were calculated at the same level of theory. Apart from the most popular Mulliken [31] derived point charges, Bader method based on partitioning of electron density distribution, Born [32] or Szigeti [33] effective charges based on charges derived from dipole-dependent properties and CHELPG [34] method based on electrostatic potential were considered. Nevertheless, for the sake of conciseness of the manuscript we selected the method of Mulliken point charges. Counterpoise correction [35, 36] was calculated to access Basis Set Superposition Error (BSSE). All calculations were performed with the GAUSSIAN 09 [37].

2.5. Fungicidal activity

Fungi strains. The antifungal activity of tested compounds was evaluated against microfungi causing mould growth phenomenon *Aspergillus niger* van Tiegen BAM 4 (ATCC 6275), *Aspergillus versicolor* BAM 8 (ATCC 11730), *Paecilomyces variotii* BAM 19 (ATCC 18502), *Penicillium funiculosum* BAM 22 (ATCC 11797) and against brown-rot fungi belonging to the phylum *Basidiomycota* (*Coniophora puteana* (Schumach).

P. Karst. BAM Ebw. 15, *Poria placenta* (Fries) Cook sensu J. Eriksson (FPRL 280) and also white-rot fungus *Coriolus versicolor* (Linnaeus) Quélet (CTB 863A).

Bioautography on thin-layer plates. The complexes of colchicine (10 mg) to be tested were dissolved in 200 µL methanol to obtain a high concentration of the solution. Portions of 10 µL volumes of tested solutions were applied as small spots on TLC plates (2 x 4 cm silica gel 60 F_{254} sheets, Merck) using micropipette. The organic solvent was evaporated by a stream of air. TLC plates with added solutions were placed in a Petri dish on the agar medium and then coated with agar medium with concentration of spores suspension in concentration of 10^6 CFU/mL, which corresponds to the logarithmic growth phase. The fungal spores were obtained from two-week agar slants. The layers were incubated for 7 days in a moist chamber with relative humidity (RH) above 95% at 28°C in the dark and the appearance of blank zones in the mycelium layer indicated antifungal activity. Fungal growth was evaluated macroscopically throughout the study period. From our previous studies we concluded that 7 days is a sufficient time for estimation of the fungal activity [38]. Each experiment was performed in triplicate, the results for each compounds were compared to the control plates. Visual evaluation of moulds growth on samples was made according to the three point scale of intensity mycelium growth:

,,-" - no visible growth under the microscope

,,±" - growth visible with the naked eye, growth of hyphae without spores

,,+" - growth visible with the naked eye, sporulation mycelium.

Agar dilution method. Complexes of colchicine (**3**, **6**) were tested against brown-rot fungi *C. puteana* and *P. placenta* and also against white-rot fungus *C. versicolor* using agar dilution methods to determine the minimal inhibitory concentration (MIC). Solutions with different chemical concentrations (from 0.0001 to 0.1g/100 mL solutions) were added into sterilized potato dextrose agar medium (PDA) prepared in Petri dishes (ø 90 mm, *h* 10 mm). Each PDA sterile plate contained 9 mL of PDA and 1 mL of an methanol solutions of complexes. For all test fungi, PDA plates without any additives were made and used as control plates. The solid plates were inoculated at the center of the Petri dish and incubated in the dark at 22°C and 70% RH. The incubation was stopped when the mycelia mass of control plates had filled the plates. The fungal growth (colony diameter) and percentage inhibition was evaluated with antifungal index (AI) calculated according to the formula AI (%)=[(A-B)/A]×100, where A and B represent the area covered with mycelium on plates, respectively.

III. RESULTS

Colchicine **1** and respective inorganic salts (Na_2CO_3, K_2CO_3, Na_2SO_4, K_2SO_4, $MgSO_4$, $CaSO_4$) in a ratio 1:1 M were dissolved in methanol and were stirred for 24h in room temperature. Compounds **2-7** were obtained as pale to dark yellow solids with very good yields. The complexes **2-7** of colchicine **1** with sodium, potassium, magnesium and calcium carbonates and sulphates were subjected to spectral studies ^{13}C NMR and ^{1}H NMR, ESI MS, MALDI MS, UV-Vis, FT IR, fungicidal activity and theoretical studies. Some of the data obtained are given in Experimental Section.

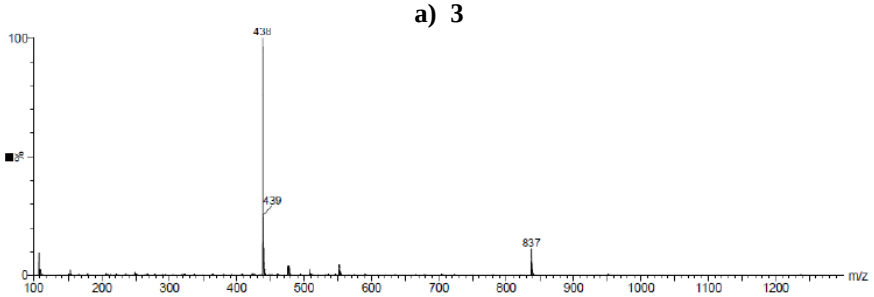
3.1 Mass spectrometry

In order to elucidate the structures of gas-phase ions derived from the complexes obtained, we applied two mass spectral techniques – electrospray ionization mass spectrometry (ESI MS), where the analyte is introduced to the instrument as a solution in polar solvent, and MALDI mass spectrometry, where the analyte is deposited on the target in the presence of an excess of additional auxiliary compound (matrix) and desorbed from the solid state to gas phase by laser beam. The first method is regarded very mild ionization way, while the latter offers somewhat harder approach.

3.1.1. ESI MS studies

The m/z signals in the ESI MS spectra of the complexes formed between colchicine **1** and monovalent or divalent cations (M = Na^+, K^+, Mg^{2+} and Ca^{2+}) at the cone voltage of 30V are given in Table 1. while the ESI MS spectra of 1:1 (M/M) complexes of colchicine with M_nX salts (M = metal, n = 1 or 2, X = carbonate or sulphate) and an example of ESI MS spectra of complexes **3** and **4** are shown in Fig. 2 a and b.

a) 3



b) 4

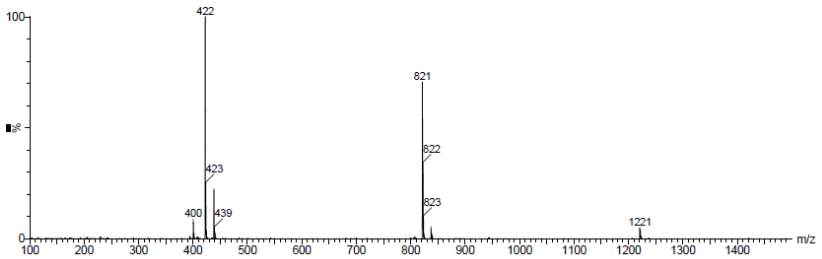


Fig. 2. The ESI MS mass spectra of colchicine complex with potassium carbonate **3** and sodium sulfate **4**

General scheme for colchicine complexes 2-7 is given in Figure 3. All spectra of the colchicine complexes with Na^+ **2**, **4** and K^+ **3**, **5** cations show signals at m/z = 422 and 438, respectively. These signals can be assigned to the respective 1:1 complexes.

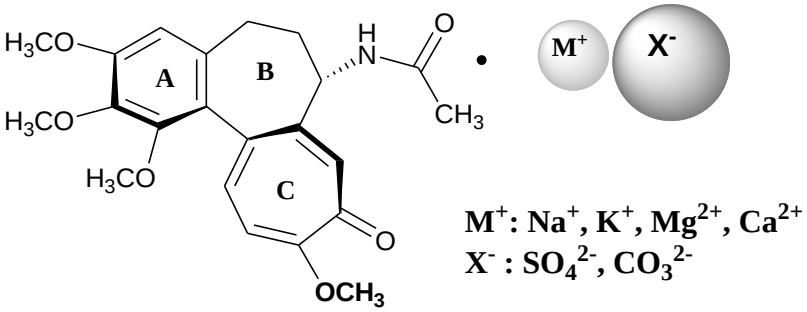


Fig. 3. General scheme for colchicine complexes **2-7**

Table 1: The main peaks in the ESI MS mass spectra of the complexes **2-7** measured at cv=30V ES^+

Mixture	m/z values					
	Comple 1:1 **[1**+ M]$^+$	Complex 1:1 [**1**+ M^{2+}-H]$^+$ **[1**+ M]$^{2+}$	Complex 2:1 [2·**1**+M]$^+$	Comple 3:1 [3·**1**+M]	Complex 2:1 [2 **1**+M]$^{2+}$	Complex 2:1 [2·**1**+M^{2+}-H]$^+$
2	422	-	821	-	-	-
3	438	-	837	-	-	-
4	422	-	821	1221	-	-
5	438	-	837	-	-	-
6	-	422	-	-	411	821
7	-	-	-	-	438	837

M^+ / M^{2+} - metal ion Na^+, K^+, Mg^{2+}, Ca^{2+}

The appearance of signals at m/z = 821 and 837 for Na^+ **2**, **4** and for K^+ **3**, **5** colchicine complexes indicates the formation of the colchicine complexes with Na^+ and K^+ cations of 2:1 stoichiometry, respectively. Unexpectedly, the ESI MS spectrum of colchicine complex with sodium sulphate **4** shows a signal at m/z = 1221, which suggests 3:1 stoichiometry. In the ESI MS spectra ES^+ there are no signals which can be assigned to the structures in which carbonate (for complexes **2** and **3**) or sulphate (for complexes **4**, **5**, **6** and **7**) anions are involved. A comparison of the relative intensities of the respective signals shows that Na^+ and K^+ cations preferentially form 1:1 complexes.

In the ES^- in ESI MS mass spectra some specific signals can be observed especially for colchicine complex with sodium sulphate **4**. For all obtained complexes **2-7** a signal at m/z = 398 which can be assigned to $[M\text{-}H^+]^-$ was observed. Moreover for **4**, **5** and **7**, other signals were observed at m/z = 797 which can be assigned to 2·**1**-H^+ and also for **4** and **5** a signal at m/z = 496 which can be assigned to **1**+R^{2-}+H^+ (R = $S0_4^{2-}$), which indicates the coordination of one colchicine molecule with one sulphate ion and proton. For complex **4** some other much more complicated complexes were observed in negative ion mode, assigned to signal at m/z = 895 and m/z = 917 indicating the formation of 2:1:1 stoichiometry complexes, 2· **1**+ R^{2-}+H^+ and 2· **1**+ R^{2-}+Na^+, respectively. For colchicine complexes with carbonates **2** and **3**, no signals of this type were observed.

In the ESI MS mass spectra both ES^+ and ES^- signals assigned to the most complicated complexes were observed for the colchicine complex with sodium sulphate **4**. In the previous study on colchicine complexes with lithium, sodium and potassium perchlorates and iodides no signals in ESI MS ES^- have been observed [16].

3.1.2.. MALDI studies

Four colchicine complexes with monovalent Na^+ and K^+ cations **2-5** were formed and investigated by MALDI mass spectrometry. Complexes **2-5** with the salts: Na_2CO_3, K_2CO_3, Na_2SO_4 and K_2SO_4, were tested in order to establish the cation and anion importance for complexing process. It should be noted that the nanospray MS of colchicine and MS^2 of the m/z 400±0.5 ion $[\mathbf{1}+H]^+$ have already been reported and representative fragment ions, which originate from m/z 400, are given, i.e. m/z 382, 368, 358, 341, 326, 310, 298 and 282. In collisionally induced dissociation MS/MS spectra of precursor ions $[\mathbf{1}+Na]^+$ and $[\mathbf{1}+K]^+$ the same m/z values of fragment ions are often found as in previously published papers, concerning the fragmentation of colchicine pseudomolecular ion $[\mathbf{1}+H]^+$. [24-26]

In the MALDI TOF MS spectra of colchicine and its complexes with alkali metal ions **2-5** with dithranol as a matrix: the ions $[\mathbf{1}+Na^+]$ and $[\mathbf{1}+K^+]$ respectively, were observed, the data (elemental composition and relative abundance) are given in Table. 2 below.

Table 2: Mass spectral data from MALDI TOF spectra (positive ion mode, matrix DIT) of colchicine complexes with sodium and potassium carbonates and sulphates **2**–**5**

Ion M = Na or K	elemental composition	**2** *m/z* (% r. a.)	**3** *m/z* (% r. a.)	**4** *m/z* (% r. a.)	**5** *m/z* (% r. a.)
$[2\cdot\mathbf{1}+M]^+$		-	-	821 (22)	837 (11)
$[\mathbf{1}+M]^+$	$C_{22}H_{25}NO_6Na$/	**422** (11)	422 (25)	**422** (25)	422 (22)
	$C_{22}H_{25}NO_6K$		**438** (42)		**438** (61)
$[\mathbf{1}+H]^+$	$C_{22}H_{26}NO_6$	400 (11)	-	-	400 (100)
$[\mathbf{1}-H_2NCOCH_3+H]^+$	$C_{20}H_{21}O_5$	341 (44)	-	341 (59)	341 (90)
	$C_{18}H_{18}O_3$	282 (44)	282 (17)	282 (75)	282 (2)
		181 (53)	181 (75)	181 (56)	
		165 (19)	166 (50)	165 (100)	
		152 (100)	152 (100)	152 (81)	

MALDI as an ionization technique is less soft than ESI, because due to the laser use, the fragmentation occurs in spite of the protective role of the matrix and, as a result, in the TOF LD^+ mass spectra of colchicine complexes with both sodium and potassium salts some fragment ions characteristic of colchicine itself are observed *m/z* = 341, *m/z*=282, *m/z* = 181, *m/z* 165, *m/z* 152.

In the TOF MS LD^+ mass spectra of colchicine complexes with potassium salts some fragment ions characteristic of colchicine itself are observed m/z= 341, m/z=282, and in the TOF MS LD^- m/z = 238/239 and m/z = 255/256. It should be noted that the complex ions containing sodium $[\mathbf{1}+Na]^+$ at *m/z* 422 appear also in the spectra of potassium complexes. The reason for this phenomenon is the ubiquity of sodium ions in the used solvents and laboratory glassware. By comparing the intensity of ions $[\mathbf{1} + Na]^+$ and $[\mathbf{1}+ K]^+$, it appears that the formation of 1:1 complexes as well as dimeric complexes $[2\cdot\mathbf{1} + M]^+$ is preferred in the presence of sulphate ions.In the negative ion mode (TOF MS LD^-) mainly the signal of an ion at *m/z* 225 (100% of relative abundance) can be seen, corresponding to the anion obtained by the deprotonation of MALDI matrix $[DIT-H]^-$. Because dithranol is used in a great excess in relation to the analyte, other anions are not detected.

To investigate the dissociation of colchicine complexes with sodium and potassium ions in collisional conditions, we performed MALDI TOF MS/MS measurements for precursor ions $[\mathbf{1}+Na]^+$, *m/z* 422 and $[\mathbf{1}+K]^+$, *m/z* 438 at collision energies of 30, 40, 50 and 60 eV. The data obtained are summarized in Table 3. There were almost no fragment ions derived from the precursors at the collision energy CE = 30 eV. At the collision energy of 40 eV, single fragment ions appear and more profound fragmentation starts at 50-60 eV. As follows from the data obtained, that colchicine complexes of potassium are not prone to collisional dissociation in general, while sodium complexes decompose to form fragments that correspond to previously reported fragments of colchicine [24-26]. Complexes of colchicine with sodium sulfate show a greater resistance to collisions than those with sodium carbonate.

Table 3: Data from MALDI TOF MSMS spectra LD^+ of colchicine complexes with sodium and potassium carbonates and sulphates (*m/z* values and % relative abundance)

	TOF MSMS LD^+ of precursor ion $[\mathbf{1}+M]^+$, M= Na, K

COMPLEX	precursor	collision energy CE [eV]		
		40	50	60
	m/z (% relative abundance)			
1+Na_2CO_3 **2**	422	282 (100)	251 (75), 225 (100), 149 (75)	-
1+K_2CO_3 **3**	438	149 (100)	149 (100)	-
1+Na_2SO_4 **4**	422	341 (100)	267 (100)	267 (89), 251 (72), 224 (100), 195 (67), 181 (50)
1+K_2SO_4 **5**	438	149 (100)	149 (100)	265 (20), 149 (100)

3.2. NMR measurements

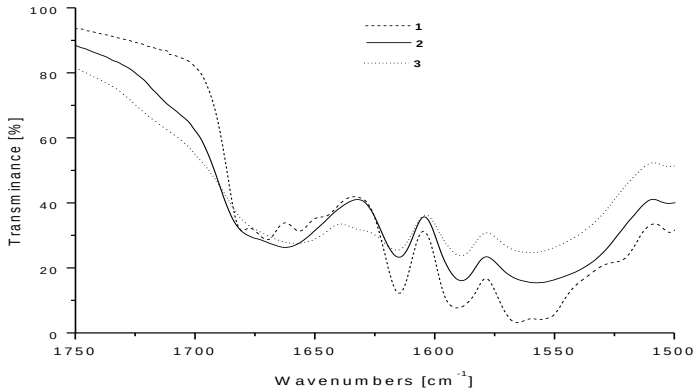
The ^{1}H and ^{13}C NMR data for the colchicine complexes **2**-**7** with sodium, potassium, magnesium and calcium sulphates and sodium and potassium carbonates in CD_3CN are given in Supporting Information in Table S1 and Table S2. In the ^{1}H NMR spectra of all obtained complexes **2**-**7**, no significant changes were detected after the complexation process. The values of chemical shifts of colchicine complexes with carbonates **2**-**3** are more or less the same like for respective cations for colchicine complexes with sulphates **4**-**7**. Some changes in chemical shifts of protons on amine group NH and at H–C8 were observed. The slightest changes in chemical shifts of hydrogen appear for amide group and the greatest change in chemical shift of this NH proton is found for colchicine complexes with Mg^{2+} **6** and Ca^{2+} **7**. Apart from these changes, these spectra give no information on the structure of respective complexes. The ^{1}H NMR spectra of colchicine and its complexes with carbonates and sulphates are almost the same, irrespective of different cations and ions (complexes **2**-**5**), which has been observed earlier for other biologically active compounds like monensin, oligomycin and their complexes with colchicine [16, 19-21].

In the ^{13}C NMR spectra of colchicine and its complexes **2**-**7** some changes in chemical shifts were observed for carbon atoms C-4a, C-7a and carbonyl groups. The chemical shifts of carbon atoms show that both carbonyl groups: at C-7 position (acetamide group) and C-9 in the tropolone ring C are involved in the coordination process.

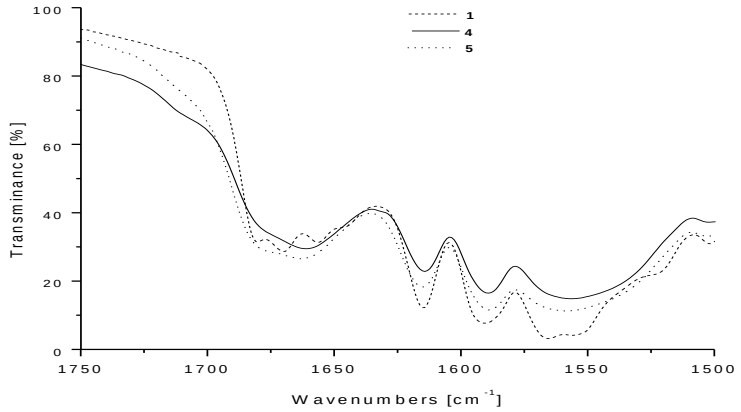
3.3. FT IR measurements

The FT IR spectra of **2**-**7** colchicine complexes with monovalent and divalent cations and for comparison the spectrum of colchicine, all in KBr pellets, are shown in Figure 4 a-c and Table 4. (in the region of carbonyl group) and full FT IR data are given in the Experimental section.

a) colchicine–sodium carbonate **2** and colchicine-potassium carbonate **3**



b) colchicine–sodium sulphate **4** and colchicine-potassium sulphate **5**



c) colchicine–magnesium sulphate **6** and colchicine-calcium sulphate **7**

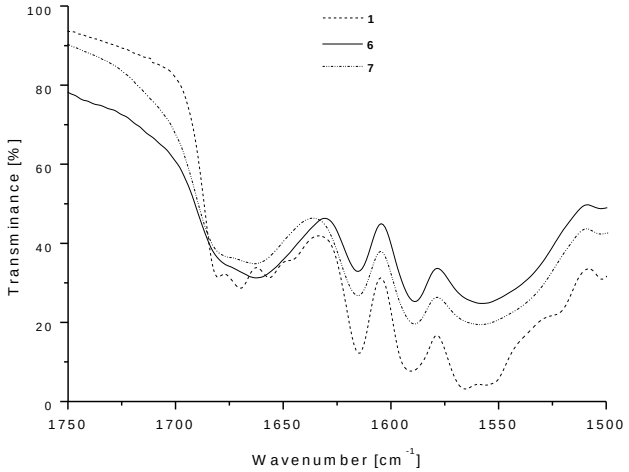


Fig. 4. FT IR spectra of colchicine **1** and its complexes **2-7** in the range of 1750-1500cm^{-1}

After the complexation process of colchicine and respective monovalent and divalent cations of sulphates and carbonates, the shapes and intensities of the bands assigned to stretching vibrations have changed. The NH stretching vibrations of colchicine is observed in the FT IR spectrum as a band with a maximum at 3359 cm^{-1}. In the FT IR spectra of colchicine complexes **2-7** the band of stretching vibration of NH is shifted towards lower wavenumbers. In the solid state colchicine exists as monohydrate [41]. The stretching vibrations of the water molecules are observed in the FT IR spectra as a band with a maximum at about 3447 cm^{-1}.The same band is also observed in the spectra of the colchicine complexes with Na^+, K^+, Mg^{2+} and Ca^{2+} cations but the maximum of the band is shifted towards lower wavenumbers in comparison to its position in the colchicine 1 spectrum at about 29-33 cm^{-1} and depends on the kind of cation and anion in the following order **5** = **7** < **3** < **4** < **2** = **6**, K^+ (K_2SO_4) = Ca^{2+} ($CaSO_4$) < K^+ (K_2CO_3) < Na^+ (Na_2SO_4) < Na^+ (Na_2CO_3) = Mg^{2+} ($MgSO_4$). FT IR spectra in the region of carbonyl group of colchicine complexes with sodium and potassium carbonates **2, 3** and colchicine complexes with sodium and potassium sulphates **4, 5** and of colchicine complexes with magnesium and calcium sulphates **6, 7** are given in Fig 4.a.b.c**,** respectively. The strongest shift to the lower wavenumbers can be observed for the band assigned to the carbonyl group on ring B from 1680 cm^{-1} for colchicine **1** to 1658 cm^{-1} for colchicine complex with potassium carbonate **3**. There were no significant changes in the shifts of the stretching vibration of carbonyl group of tropolone ring C in the spectra of all colchicine complexes **2-7**, which means that this part of colchicine molecule is not involved in the complexation process as much as the carbonyl group on tropolone ring C.

Table 4: Wavenumbers of colchicine complexes **2-7** in the region of carbonyl group (in KBr pellets)

Assignments	Wavenumbers [cm^{-1}]						
	1	**2**	**3**	**4**	**5**	**6**	**7**
υC=O	1680	1661	1663	1662	1663	1662	1658
υC=O	1615	1614	1615	1615	1615	1615	1616

After the complexation process, in the FT IR spectra of all obtained complexes **2-7** the bands assigned to the stretching vibration of υC=C at 1670 cm^{-1} and 1656 cm^{-1} (observed in the FT IR spectrum of colchicine **1**), disappear.

The stretching vibrations of carbonates usually appear as a very strong band in the range of 1450-1410 cm^{-1} and the second band with medium intensity in the range 880-860 cm^{-1}. This second band position is shifted to lower wavenumbers with increasing of atomic weigh of the cation. This correlation was also observed for complexes **2** with Na^+ cation (841cm^{-1}) and **3** with K^+ cation (832cm^{-1}). Many carbonates also show absorption in the 750-710 cm-1 region. In addition, the number of bands between 1110cm^{-1} and 700 cm^{-1} increases in most cases from two to five whilst the bonded OH grouping are evident at 3300cm^{-1}.

Sulphate anions are responsible for a very strong band corresponding to stretching vibration in the range 1130-1080 cm^{-1} accompanied by a considerably weaker band in the region 680-610 cm^{-1}. For the calcium sulphate dihydrate salt the strong band was centered at 1140cm^{-1} but in the spectrum of the pure calcium sulphate salt a group of these bands was found in this region, which means that both hydration and crystal symmetry may influence the spectra.

3.4. Theoretical studies

Eight different interaction schemes in the colchicine complexes with sodium cation suggested by experimental measurements were subjected to further computational studies. Figure 5 presents initial interaction schemes for 1:1 complexes (structures **A**-**C**), 2:1 (structures **D**,**E**) and 3:1 (structures **F**-**H**).

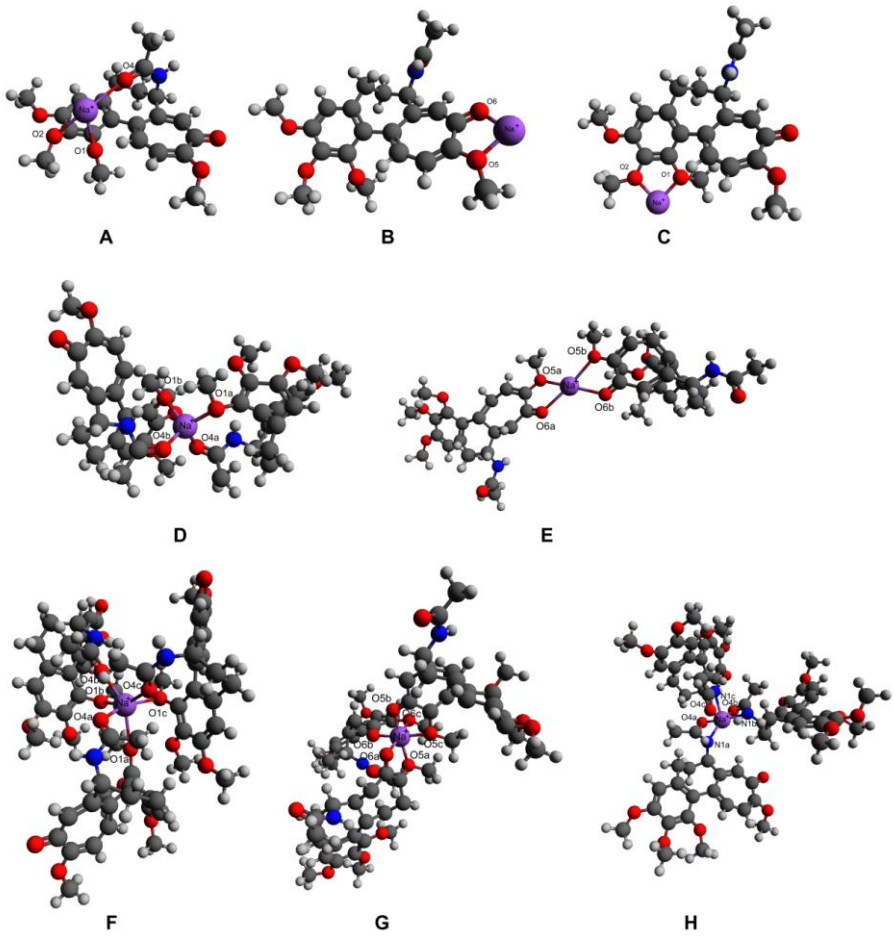


Fig. 5. Initial structures for interaction schemes of 1:1, 2:1 and 3:1 stoichiometry of colchicine complexes with Na^+.

The interaction energies calculated for each type of colchicine complexes with sodium cation are shown in Table 5.

Table 5: The interaction energies (counterpoise corrected and uncorrected) calculated for the studied interaction schemes of colchicine with Na^+ cation.

Colchicine complex	Counterpoise interaction Energy (uncorrected) [kcal/mol]	Counterpoise interaction Energy (corrected) [kcal/mol]
1:1 Type **A**	-82.7	-80.3
1:1 Type **B**	-68.5	-67.0
1:1 Type **C**	-53.6	-52.4
2:1 Type **D**	-127.2	-119.4
2:1 Type **E**	-117.7	-114.2
3:1 Type **F**	-165.7	-151.1
3:1 Type **G**	-142.1	-134.1
3:1 Type **H**	-144.1	-133.8

Absolute energy baseline [Hartree]: -1520.506867[A], -1520.492234[B], -1520.466755[C], -2878.90264[D], -2878.887362[E], -4237.299946[F], -4237.143139[G], -4237.244115[H]

For 1:1 stoichiometry complexes of colchicine with sodium cation the most favorable interaction energy (-80.3 kcal/mol) had **A** structure. Other interaction schemes were less energetically favorable (see Figure 6).

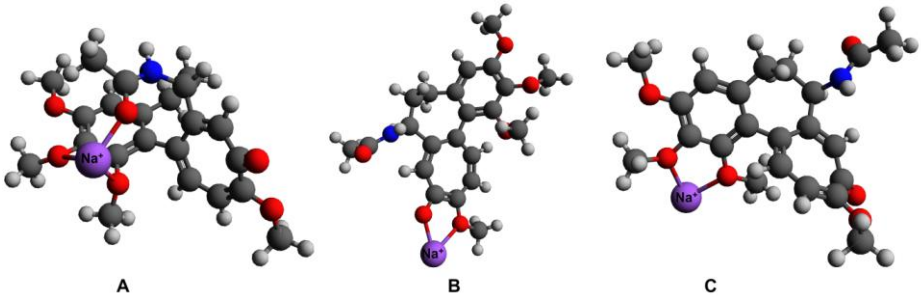


Fig. 6. The optimized structures of 1:1 stoichiometry structures of colchicine with sodium cation.

For the complexes of 2:1 stoichiometry, the most favourable interaction energy (-119.4 kcal/mol) was found for structure **D** which was most probably additionally stabilized by weak interaction between Na^+ and O1 colchicine oxygen atom (Na…O1 distance 2.542Å). The optimized structures of 2:1 stoichiometry complexes are shown in Figure 7.

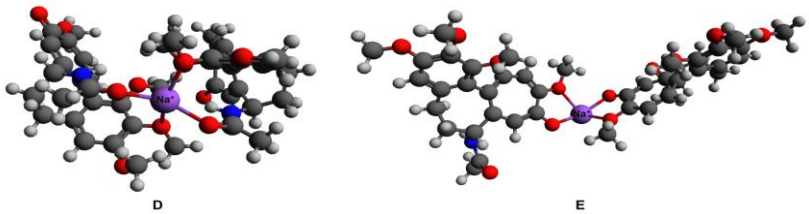


Fig. 7. The optimized structures of 2:1 stoichiometry complexes of colchicine with sodium cation

From among the colchicine structures of 3:1 stoichiometry the most favorable interaction energy (-151.1 kcal/mol) was found for **F** structure. Two of colchicine ligands coordinated sodium cation via O4 oxygen atom only but one of colchicine molecule interacted more strongly as it used O1, O2 and O4 oxygen atoms to bind central Na^+ cation. An attempt to obtain a structure with Na^+ coordinated by N1 nitrogen atom of colchicine failed, because during the optimization the coordination mode was changed to Na^+...O instead of the Na^+...N. . The structures of the optimized 3:1 stoichiometry complexes are shown in Figure 8.

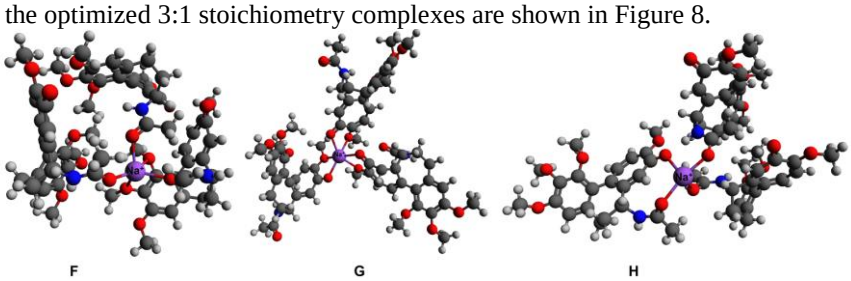


Fig. 8. The optimized structures of 3:1 stoichiometry complexes of colchicine with sodium cation.

Table 6: Selected geometry parameters and calculated Mulliken partial charges for each interaction schemes of colchicine complexes.

Colchicine complex	Sodium cation partial charge [e^-]	Coordinating atom	Coordinating atom partial charge [e^-]	Distance between the coordinating atom and the cation [Å]
1:1 Type **A**	+0.835	O4	-0.460	2.163
		O1	-0.467	2.234
		O2	-0.424	2.663
1:1 Type **B**	+0.836	O5	-0.424	2.381
		O6	-0.467	2.107
1:1 Type **C**	+0.876	O1	-0.506	2.255
		O2	-0.526	2.179
2:1 Type **D**	+0.636	O4[a]	-0.422	2.329
		O1[a]	-0.415	2.542
		O2[a]	-0.428	2.414
		O4[b]	-0.448	2.205
		O2[b]	-0.424	2.626
2:1 Type **E**	+0.775	O5[a]	-0.421	2.340
		O6[a]	-0.475	2.202
		O5[b]	-0.420	2.344
		O6[b]	-0.475	2.196
3:1 Type **F**	+0.596	O4[a]	-0.388	2.364
		O1[a]	-0.420	2.485
		O2[a]	-0.424	2.569
		O4[b]	-0.435	2.209
		O4[c]	-0.420	2.363
3:1 Type **G**	+0.669	O5[a]	-0.405	2.401
		O6[a]	-0.416	2.340
		O5[b]	-0.428	2.379
		O6[b]	-0.371	2.339
		O5[c]	-0.401	2.388
		O6[c]	-0.297	2.395
3:1 Type **H**	+0.683	O4[a]	-0.421	2.344
		O6[a]	-0.449	2.379
		O4[b]	-0.501	2.209
		O4[c]	-0.437	2.299

[a, b, c]- atoms from a, b or c colchicine molecules

Selected interatomic distances and Mulliken point charges are shown in Table 6. Amongst the most favorable interaction schemes the Na^+…O4 distance is the shortest one (2.163Å for **A** 1:1 stoichiometry, 2.205Å for **D** 2:1 stoichiometry and 2.209Å for **F** 3:1 stoichiometry) suggesting that it is the strongest interaction between the central Na^+ cation and colchicine ligand. Mulliken partial charges on sodium cation varied from +0.595 e^- in structure **F** of 3:1 stoichiometry to +0.876 e^- in structure **C** of 1:1 stoichiometry. The Mulliken point charges calculated for coordinating oxygen atoms varied form -0.526 e^- for O2 oxygen atom in **C** structure of 1:1 stoichiometry to -0.297 e^- for O6 oxygen atom in 3:1 stoichiometry **G** structure.

3.5. Fungicidal activity of colchicine complexes

Colchicine binds with lower affinity to fungal tubulins as compared to mammalian tubulins [40]. Colchicine has been tested previously against antifungal activity for *Aspergillus niger*, *Allomyces javanicus*, *Aspergillus spp*., *Butrylis cinerea*, *Caprinus radians*, *Diaparhte perniciosa*, *Mucor sp*., *Penicillium notatum*, *Psilocyte semilanceolata*, *Dia* and *Saccharomyces cerversiae*, *Candida* [41, 42]. In some cases changes were not observed but in for others were, for example prevention of candida formation or inhibition of fungi growth.

Colchicine is effective in gout therapy in the treatment of acute gout. It has been established that gout in human body in most cases is induced by the fungal species *Ustilago maydis*, *Chaetomium trilaterale*, *Saccharomyces cerversiae* and yeast *Candida utilis* and fungal metabolites cyclosporine, ergotamine and penicillin. Beer and wine are produced with involvement of fermentations of *Saccharomyces cerversiae* so drinking them is tantamount to drinking a fungal culture. Moreover in beer there are large amounts of uric acid which cause tophi [42]. Colchicine significantly inhibited the fungicidal activity of neutrophils against *Penicillium marneffei* in dose-dependent manner and this inhibition was not due to its cytotoxic effect [43].

In this study the properties of derivatives of colchicine against microfungi were analyzed using the bioautography-TLC method. This method belongs to microbiological screening tests commonly used for identification of fungistatic properties of compounds. All salts constituting complexes with colchicine were also tested and they were found not to show antifungal properties.

The results for the new derivatives of colchicine are presented in Table 7. The fungal mycelium growing all over the control plates evidenced that it was in good condition during the test. The results permit a conclusion that the antifungal properties of some new complexes of colchicine may be potentially useful for controlling moulds. Bioautography combined with TLC showed that the growth of all *Aspergillus* strains was inhibited by complex **4** forming clear inhibition zones, though the other species exhibited weak growth. The tested compounds **3**, **5, 7** in the first days of the test, inhibited the growth of mycelium *A. niger* but in the following days of the test they were inactivated (not shown in the table). Derivative **3** besides showing fungistatic properties against *P. variotii* also in the first days of the test inhibited the growth of *A. versicolor*. No antifungal properties were observed for compound **2**. Moreover, the above compounds also complex **6** showed very high fungistatic activity against *P. funiculosum* and in addition inhibited the growth of *A. versicolor* and *P. variotti* in the first days of observation.

Table 7: The results of bioassay tests against microfungi

Colchicine and complexes	Fungal species			
	A. niger	*A. versicolor*	*P. variotii*	*P. funiculosum*
1	+	+	+	+
2	+	+	+	+
3	+	+	**-**	+
4	**-**	**-**	+	+
5	+	+	+	+
6	+	+	+	**-**
7	+	+	**-**	+
control plate	+	+	+	+

\- no visible growth under the microscope
± growth visible with the naked eye, growth of hyphae without spores
\+ growth visible with the naked eye, sporulation mycelium

MICs of colchicine complexes **3** and **6** against *C. puteana*, *P. placenta* and C. *versicolor* were determined by the agar dilution method. The MIC was defined as the lowest concentration of a given agent at which no visible growth of fungi was noted or the lowest concentration that inhibited more than 80% of growth. The *in-vitro* susceptibility testing using complexes of colchicine is shown in Table 8. It has been proven that compound **3** has inhibitory effects against white rot fungi *P. placenta* and *C. versicolor* and also against brown rot fungus *C. puteana*. Compound **6** showed only fungistatic properties against one fungus *P. placenta*. Among the *Basidiomycota* fungi, the MICs of compound **3** were within the range of 0.1-0.01 g/mL for *C. puteana* and *C. versicolor* while both compounds **3** and **6** had a MIC equal to 0.1 g/mL for *P. placenta*. Comparison of MICs values for the fungi tested revealed a difference in the tolerance against the tested compounds. *P. placenta* showed greater tolerance with MICs being one order of concentration magnitude higher than the values determined for *C. puteana* and *C. versicolor* within the selected concentration range for compound **3.** *P. placenta* did not show such a high resistance as above towards compound 6, while the other fungi proved to be more resistant to this compound.

Table 8: The results of bioassay tests for minimal inhibitory concentration (MIC) against *Basidiomycota* fungi

Compound	Fungi	Antifungal index of tested compounds against *Basidiomycota* fungi[a] [g/mL solution]			
		0.1	0.01	0.001	0.0001
3	*P. placenta*	86.90±4.40	71.22±9.80	27.26±4.77	24.02±5.94

	C. puteana	100±0.00	50.34±3.79	34.32±6.81	32.04±5.86
	C. versicolor	100±0.00	45.21±3.29	23.57±2.72	18.72±9.20
6	*P. placenta*	100±0.00	0.56±0.23	0.00±0.00	0.00±0.00
	C. puteana	56.06±3.51	14.79±1.69	16.91±1.28	10.91±1.29
	C. versicolor	26.86±7.20	1.56±0.53	0.57±0.50	0.00±0.00

[a] Data are presented as the means ± relative standard deviation (RSD) of five determinations

On the basis of the results from antifungal assays we can conclude that fungistatic activity of the new complexes of colchicine may be potentially useful for controlling the growth of fungi. The tested compounds inhibited the growth of some types of microfungi, but there was no compound active against all tested species of moulds. However, complex **3** demonstrated fungistatic properties against rot fungi belonging to the phylum *Basidiomycota.* Nevertheless, the approach developed in the study could be a useful way to identify a new class of antifungal agents.

IV. CONCLUSION

Series of new colchicine complexes with monovalent and divalent metal cations of carbonates and sulfates were obtained and described by spectral analysis. In the present work it was found that colchicine can form stable complexes with ions of human body fluids like: Na^+, K^+, Ca^{2+} and Mg^{2+}. In the our previous work it was found that colchicine forms complexes with lithium, sodium and potassium iodides and perchlorates mainly in stoichiometry 1:1 and less preferable in 2: 1 stoichiometry. In comparison to the in present study it was found that colchicine with sodium, potassium, magnesium and calcium sulfates and sodium, potassium carbonates can form much more complicated complexes in stoichiometry 2:1 and for complex with sodium sulphate 3:1 and 2:1:1. Moreover, sulphate anions were also involved in complexation process.

Quantum-mechanical calculations helped indicate which colchicine atoms are involved in coordinating sodium cation. It appears that one colchicine molecule is particularly strongly bound to Na^+ and interacts via O4, O1 and O2 oxygen atoms. Moreover, our calculations suggest that Na^+…O4 interaction is energetically favored for complexes stoichiometry of 1:1, 2:1 and 3:1. It also seems that nitrogen atom of colchicine is not favored as a donor when compared with oxygen atoms.

New colchicine complexes show fungicidal activity against selected species of moulds, in the future they may be potentially useful for controlling the growth of fungi.

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